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ARTICLES

Older male reed buntings are more successful at gaining
extrapair fertilizations

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The importance of extrapair paternity (EPP) as an aspect of mixed reproductive strategies is currently the focus of many studies. Since females have at least some control over the occurrence of inseminations, they are expected to engage in extrapair copulations only if they benefit, for instance through gaining high-quality or compatible genes for their offspring, or insurance against infertility. To distinguish between these benefits, we studied characteristics related to intermale variation in fertilization success as well as differences in fitness between half-siblings in the reed bunting, *Emberiza schoeniclus*, a socially monogamous passerine with high levels of EPP (50% of young). We found that older males were more successful at siring offspring in other broods and a nonsignificant tendency for them to be less cuckolded in their own broods. The success of older males does not support the fertility insurance hypothesis: a male's sperm storage capacity did not predict his fertilization success, the occurrence of infertile eggs was unrelated to the occurrence of EPP in a nest and older males had more infertile eggs in nests with no EPP. Extrapair young had longer tarsi at fledging than their maternal half-siblings, but we found no other differences between maternal or paternal half-siblings in several presumed quality traits. Owing to the absence of long-term fitness data, we are unable to determine whether the difference in tarsus length is related to a difference in fitness benefits. At this time, we cannot exclude the possibility that females do gain genetic benefits through EPP.

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With molecular techniques becoming widespread, the study of extrapair mating behaviour in socially monogamous species has become the main focus of many behavioural ecologists. Extrapair paternity (EPP) has been found to occur in 86% of socially monogamous passerine species studied so far (Griffith et al. 2002). Males may increase their reproductive output without additional paternal investment by engaging in extrapair copulations (EPCs), as these can lead to extrapair fertilizations (EPFs; Westneat et al. 1990; Birkhead & Møller 1992). Since reproductive success for females is limited by the number of eggs produced, the benefits of EPCs to females are less clear. Given that EPCs are considered to be costly, that females have at least some control over whether insemination occurs (Birkhead

& Møller 1992) and that in some species females actively seek EPCs (reviewed in Westneat & Stewart 2003), females are expected to engage in EPCs only if there are potential benefits (Westneat et al. 1990; Birkhead & Møller 1992).

Females may obtain 'good genes' for their offspring through mating with a high-quality extrapair partner (Westneat et al. 1990; Birkhead & Møller 1992). Male characteristics that reflect quality are expected to be related to fertilization success: a positive relation between male fertilization success and certain male characteristics, such as age, size, plumage characteristics and song (reviewed in Griffith et al. 2002), is often interpreted as support for females obtaining genetic benefits for their offspring. However, a different explanation for this relation is based on the possible association of functional fertility of a male (i.e. the success of ejaculates in fertilizing eggs) with preferred male characteristics (Sheldon 1994). Sheldon (1994) argued that females may seek EPCs as a direct benefit to themselves,

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namely to insure against functional infertility of their partner, resulting in the same patterns of fertilization success as would be found when females seek genetic benefits for their offspring. One way to distinguish between these two hypotheses is to determine whether there are differences between offspring sired through EPCs and offspring sired through within-pair copulations (Sheldon 1994; Griffith et al. 2002). If females gain genetic benefits for their offspring, then extrapair young (EPY) are expected to be fitter than within-pair young (WPY) in the same brood (i.e. maternal half-siblings). As fitness of offspring is often difficult to measure directly, factors have been examined that are expected to be related to fitness. EPY have been shown to have a higher survival to fledging, a better body condition at fledging, a higher postfledging survival and a stronger immune response (reviewed in Griffith et al. 2002). However, several other studies showed no difference between WPY and EPY (reviewed in Griffith et al. 2002).

In addition to the intrinsic effects of paternal genes (i.e. good genes), the importance of the interaction between maternal and paternal genes (i.e. genetic compatibility; reviewed in Tregenza & Wedell 2000) has been assessed by using extrapair offspring. Heterozygosity reflects the individual level of genetic diversity and is often related to reproductive success (reviewed in Hansson & Westerberg 2002); therefore, females may benefit through seeking copulations with genetically dissimilar mates (Tregenza & Wedell 2002; Richardson et al. 2004; but see Hansson et al. 2004). In blue tit, *Cyanistes caeruleus*, broods of mixed paternity, EPY were more heterozygous than their maternal half-siblings (Foerster et al. 2003). Extrapair bluethroat, *Luscinia svecica*, offspring not only had higher immunocompetence than their maternal half-siblings in the same brood, but also higher immunocompetence than their paternal half-siblings reared in the male's own nest (Johnsen et al. 2000). Johnsen et al. (2000) therefore concluded that female bluethroats were seeking compatible genes for their offspring rather than just good genes.

We studied extrapair mating behaviour and its fitness consequences in a population of reed buntings, *Emberiza schoeniclus*, in The Netherlands. The reed bunting is a small (18 g), sexually dimorphic, socially monogamous passerine. Males have a black head and throat patch ('badge') with a contrasting white collar, and can often be seen singing high on reed stems or small bushes. Females lack these conspicuous traits, and are also more cryptic in their behaviour. The levels of EPP found in different populations are among the highest found in socially monogamous passerines (55% in England: Dixon et al. 1994; 50% in The Netherlands: Bouwman et al. 2005). Three types of data are required to determine how females may benefit from EPP: (1) the distribution of EPP among broods; (2) the distribution of EPP among males with different characteristics; and (3) fitness differences between WPY and EPY in broods of mixed paternity (Griffith et al. 2002). Previously, when addressing the first type of question, we found that EPP was not evenly distributed among broods: more broods than expected contained no EPY ($N = 68$ nests; Bouwman et al. 2006). Our aim in this study was to address the second and third types of question. First, we investigated whether male characteristics, such as age, biometrics, plumage characteristics, song

quality and quantity, and sperm storage capacity, were related to within- and extrapair fertilization success. Second, we investigated whether there were differences in biometrics, heterozygosity, hatching success and fledging success between WPY and EPY. If females seek good or compatible paternal genes for their offspring, EPY are expected to be 'better' than WPY in broods of mixed paternity.

METHODS

General

From 2001 to 2003 we studied a population of reed buntings in a 13-ha study site, on the island of Noorderplaat (45 ha) in 'De Biesbosch' National Park in the Netherlands (51°45'N, 4°45'E). The study site had an approximate density of 3 pairs/ha. For a full description of the study site and general methods see Bouwman et al. (2005). In brief, adult reed buntings were caught with mist nets and ringed with a numbered aluminium ring and a specific combination of three colour rings for individual recognition. K.M.B. was licensed by the Dutch Bird Ringing Centre to catch and ring reed buntings and was given permission by the State Forestry Service (staatsbosbeheer) to catch and ring in 'De Biesbosch' National Park. Birds were measured and a blood sample (approximately 20 µl) was taken from the brachial vein for DNA analysis (under licence of the Animal Experiment Committee of the University of Groningen) and stored in 96% ethanol at room temperature. We determined the identities of the male and female belonging to a nest (territorial birds) by direct or video observations of colour-ringed birds protecting the nest, incubating and feeding nestlings. In 2001 approximately 50% of adults were sampled, while in 2002 and 2003 nearly all adults were sampled (2002: 98%, $N = 88$; 2003: 94%, $N = 72$).

Nests are built on or just above the ground and we located them through systematic searches that flushed females off the nest, or by observing territorial birds for any nest-related activities. Two days after nestlings hatched, we took a small blood sample (approximately 10 µl) from their leg vein (under licence of the Dutch ethical committee). If eggs did not hatch, we inspected them for embryonic development, which, if present, was used as a source of DNA. In 81% of nests with unhatched eggs ($N = 47$), the unhatched eggs were rotten or showed no development. In these cases we assumed that the eggs were infertile, although embryo mortality may have occurred at an early stage. At 7 days of age the nestlings were ringed and measured. In total we found 94% of nests ($N = 153$) before hatching. Of these 144 nests, 31% were predated before hatching. None of the remaining nests ($N = 100$) showed complete hatching failure. It thus appears unlikely that we failed to find many nests with complete hatching failure. Within our study site we found 97.4% of all nests that fledged young ($N = 78$) in 2002 and 2003; in only two cases did we see fledglings without locating the nest. However, owing to high levels of predation, we were unable to locate all nests in the study area. As there was no obvious gradient in risk of predation

across the site, we believe we obtained a random sample of individual reproductive success for all males in our site. The study was carried out under licence of the Animal Experiment Committee of the University of Groningen.

Male Morphological Measurements

We measured adult tarsus length with callipers (± 0.1 mm), wing length with a stop ruler (± 0.5 mm) and body mass with a 30-g Pesola spring balance (± 0.1 g). We took two digital pictures of the badge, together with a ruler, while the male was positioned in a standardized way. In between the two pictures, the bird was allowed to relax. To extract the size of the badge from the picture we used Adobe Photoshop 5.0.2 (Adobe Systems Incorporated, San Jose, U.S.A.); we compared the number of pixels in 1 cm^2 to the number of pixels within the selected area covered by the badge. The repeatability of badge size for a single male within one capture event was high (Lessells & Boag 1987; Table 1). The colour of the badge ranged from brown (caused by brown fringes on the edge of the black badge feathers in winter plumage) to completely black (full breeding plumage; Cramp & Perrins 1994). The colour of the badge was compared by the same observer with five categories of pictures of reed bunting museum skins, and given a score on a scale of 1 (brown) to 5 (completely black). The badge colour was highly correlated with time of season (Spearman correlation: $r_s = 0.72$, $N = 92$, $P < 0.001$), as was also found by Dixon (1993), and the time between measurements of the same individual was highly correlated with the difference in colour score ($r_s = 0.79$, $N = 46$, $P < 0.001$). We therefore restricted the measurements for badge colour to the peak of the breeding season, between 20 April and 30 June. There was no significant relation between badge colour and day within this period ($r_s = 0.21$, $N = 32$, $P = 0.26$). Neither was there a difference in return rates the following season between males in relation to their badge colour as measured in the peak of the breeding season (score 1: $N = 0$; score 2 + 3: 75% ($N = 8$); score 4 + 5: 58% ($N = 24$); Fisher's exact test: $P = 0.68$). We estimated the repeatability of measurements using 46 males that were captured on more than one occasion within a year (Lessells & Boag 1987; Table 1). As there

was a significant relation between mass, day and time of day ($F_{2,110} = 4.72$, $R^2 = 0.080$, $P = 0.011$), we used the unstandardized residuals of the regression as a measure for mass.

For each male, we measured the cloacal protuberance, an anatomical structure that results from the enlargement of the seminal glomerus (i.e. the site of sperm storage) during the reproductive season (Lake 1981), which reflects sperm storage capacity (Nakamura 1990; Birkhead et al. 1991, 1993). As it approximates a barrel shape, we measured the height and width of the cloacal protuberance, with callipers (± 0.1 mm), from 1 April onwards, and we calculated its volume (mm^3) as the height $\times \pi$ (width/2)² (Mulder & Cockburn 1993). There was a significant curvilinear relation between day of season and cloacal protuberance ($F_{2,72} = 72.2$, $R^2 = 0.67$, $P < 0.001$), so we used the unstandardized residuals of the regression as a measure for the cloacal protuberance. As we measured it twice for only six individuals within one season, we did not calculate the repeatability for this characteristic.

Age and Survival

In 2002 and 2003, on average 96% of adult individuals within the study area were ringed (2002: $N = 88$; 2003: $N = 72$) and approximately 80% of individuals from territories surrounding our study area were ringed ($N = 20$). On average 42% of ringed adults breeding in 2002 returned to our study area the following breeding season and reoccupied the same or a nearby territory. As recruitment rates of fledglings were very low and adult reed buntings could not be reliably aged from plumage characteristics, few adults were of known age. We assumed ringed adults returning in 2003 to be 'old' breeders and unringed adults to be 'young' breeders (following e.g. Veiga 1993; Yezerinac & Weatherhead 1997). This classification was supported by our measurements of wing length and badge colour, as wing length and scores for badge colour increased with age and 'old' males had longer wings and blacker badges than 'young' males (see Results).

Song Recording and Analysis

Reed bunting males sing two types of song: a fast, premating song and a slow, postmating song (Nemeth 1996). The first is sung mostly during territory settlement and the second during the incubation period of the social female (Nemeth 1996). Mated males sing throughout the day, but a peak in singing is reached approximately 1.5 h before sunrise and a second at 6–7 h after sunrise (approximately 1100 hours, GMT + 2 h; O'Malley 1993; personal observation). We collected data on both song quantity and song quality. First, we estimated song quantity by visiting all territories two times daily ($N = 74$ observations per territory) and checking whether the territorial male was singing or not. Based on these observations, we calculated the percentage time spent singing. Second, we measured song quality by recording song of 29 mated males in May and June 2003, using a Marantz portable cassette recorder (model PMD 222) and a Sennheiser directional microphone (model ME66/K6). Recordings were made throughout the day, whenever an identified male

Table 1. Repeatabilities (r) of male reed bunting characteristics

Characteristic	F	df (between, within)	r
Morphometrics			
Mass (residual)	6.37*	42, 43	0.73
Wing length	21.89*	45, 38	0.91
Tarsus length	38.03*	45, 38	0.95
Badge size (within capture)	11.47*	196, 197	0.84
Badge size (between captures)	3.69*	45, 37	0.57
Song quality measures			
Song length	3.70*	27, 127	0.33
Pause length	5.29*	27, 127	0.44
%Performance time	3.47*	27, 127	0.31
Syllable number	7.35*	27, 127	0.54

* $P < 0.001$.

was singing. The mean total recording time for each male \pm SE was 29.5 ± 0.9 min (range 17.4–39.8 min). On average 5.4 ± 0.4 (range 1–11) song bouts were recorded per male; a bout was included when it consisted of a minimum of 20 songs. Tape recordings were analysed with Avisoft SASLab Pro version 4.23b for Windows (Avisoft Bioacoustics, Berlin, Germany) following Nemeth (1996).

We measured the length of each song (± 0.01 s), the length of the pause between songs (± 0.01 s), the percentage performance time (i.e. song length/(song length + pause length)), the number of syllables within a song and the total number of different syllables produced by a male (repertoire size). Syllables were classified by eye from sonagrams. Song length, syllable number and percentage performance time were highly correlated (Pearson correlation: $r_{27} > 0.54$, $P < 0.003$) and pause length and percentage performance time were highly correlated ($r_{27} = -0.75$, $P < 0.001$). We calculated the repeatability of these measures between different bouts of the same male (Lessells & Boag 1987; Table 1). As syllable number and pause length showed the highest repeatability and were not significantly correlated with each other (Table 2), we selected these variables from the parameters of song quality for inclusion in further analyses. Although the total recording time differed between males, we found no relation between the number of different syllables produced by a male and the total recording time ($r_{27} = 0.23$, $P = 0.24$). We therefore used the total number of different syllables produced by a male as an estimate for repertoire size. This measure showed no correlation with any of the other song quality measures (all $P > 0.17$).

Nestling Characteristics

We weighed and measured 352 nestlings from 94 nests on several occasions between 0 (day of hatching) and 10 days after hatching. Final measurements were usually taken at day 7, as the risk of nestlings leaving the nest prematurely increased after this time (normal departure: day 10–12). Up to approximately day 3 after hatching, nestlings were only weighed (± 0.1 g) with a Pesola spring balance of 10 or 30 g. From day 4 to day 10, nestlings were weighed and tarsus length was measured with callipers (± 0.1 mm).

Four indexes of heterozygosity (i.e. individual heterozygosity, standardized heterozygosity, internal relatedness and mean d^2) were used as measures of degree of genetic diversity. These indexes were related to measures of fitness in other species (Coulson et al. 1998; Amos et al. 2001; Hansson et al. 2001; Foerster et al. 2003). Individual heterozygosity was defined as the proportion of scored loci that were heterozygous, while standardized heterozygosity was calculated by dividing individual heterozygosity by the average heterozygosity of the typed loci (Coltman et al. 1999). We calculated the internal relatedness (i.e. comparison of two alleles at each locus for each individual) so that the sharing of rare alleles was weighted more than the sharing of common alleles (Amos et al. 2001). The mean d^2 was calculated as the squared distance in repeat units between the two alleles, averaged for all scored loci (Coulson et al. 1998). In total, 98% of sampled offspring ($N = 501$) were scored at six microsatellite loci,

Table 2. Correlations between characteristics of males

	Mass (residual)	Wing length	Tarsus length	Badge size	Badge colour	%Time spent singing	Syllable no.	Pause length	Repertoire size
Wing length	0.39** (88)	—							
Tarsus length	0.40** (88)	0.22* (88)	—						
Badge size	0.41** (88)	0.41** (88)	0.03 (88)	—					
Badge colour	0.16 (44)	0.48** (47)	0.36* (47)	0.37* (47)	—				
%Time spent singing	0.25 (30)	0.35† (30)	0.21 (30)	0.19 (30)	0.39† (24)	—			
Syllable no.	0.21 (28)	−0.01 (28)	−0.22 (28)	−0.15 (28)	−0.33 (22)	−0.03 (26)	—		
Pause length	−0.14 (28)	−0.10 (28)	−0.12 (28)	−0.19 (28)	−0.16 (22)	−0.22 (26)	0.34† (28)	—	
Repertoire size	−0.6 (28)	−0.12 (28)	−0.17 (28)	−0.18 (28)	−0.21 (22)	−0.1 (26)	0.05 (28)	−0.07 (28)	—
Cloacal protuberance	0.29* (49)	0.34* (49)	0.14 (49)	0.18 (49)	0.24 (39)	0.37 (18)	0.31 (16)	−0.17 (16)	0.35 (16)

The correlation coefficient, sample size (in parentheses) and statistical significance are shown. † $P < 0.1$; * $P < 0.05$; ** $P < 0.01$.

while the remaining 2% were scored at five loci (see [Nestling Sex Determination and Paternity Analysis](#) below). As was found by [Foerster et al. \(2003\)](#), individual heterozygosity, standardized heterozygosity and internal relatedness were all highly correlated with each other (all: $R > 0.97$, $P < 0.0001$), and all three measures generated similar results. Since [Amos et al. \(2001\)](#) argued that internal relatedness generally explains most variation, we chose to present the results of this variable. Mean d^2 was less strongly correlated with the other genetic diversity measures (all $R < 0.29$, $P < 0.001$).

Nestling Sex Determination and Paternity Analysis

We extracted DNA from blood samples by using salt extraction ([Richardson et al. 2001](#)). Nestlings were sexed with Griffith's universal PCR method for the sexing of birds ([Griffiths et al. 1998](#)). Individuals were genotyped with six fluorescently labelled microsatellite markers: *Escμ1*, *Escμ4*, *Escμ6* ([Hanotte et al. 1994](#)), *Pdμ5* ([Griffith et al. 1999](#)), *Mcyμ4* ([Double et al. 1997](#)) and *Ppi2* ([Martinez et al. 1999](#)). Parentage was determined by using a likelihood-based approach in CERVUS (version 2.0; [Marshall et al. 1998](#)). This program assesses the confidence of paternity assignment by using criteria generated through a simulation that take into account allele frequencies in the population, the number of possible candidate parents, the proportion of candidate parents sampled, and the percentage of missing genetic data and genotyping errors. The criteria (delta criteria) estimate the critical difference between the LOD scores (the natural logarithm of the likelihood ratio) of the first and second most likely candidate at a level of >95% confidence and >80% confidence. None of the loci deviated significantly from Hardy–Weinberg equilibrium. Using the observed allele frequencies, CERVUS calculated a total exclusionary power for the six microsatellite loci; in both years the probability of exclusion was 0.993 for assigning the father when the mother is unknown ('first parent') and 0.999 for assigning the father when the mother is known ('second parent'). In 2 years 501 offspring were genotyped from 129 nests. Paternity was assigned to 90% of all offspring with 80% confidence and to 87% with 95% confidence. Extrapair males sired 54.6% of offspring in 2002 ($N = 262$) and 46.4% of offspring in 2003 ($N = 211$). The genetic father was identified for 91.3% of extrapair offspring ($N = 241$).

Data Analyses

Male fertilization success

We investigated the relation between male characteristics and male fertilization success from three different points of view. First, we tested whether there was a relation between the proportion of EPP in a male's own brood ('cuckoldry rate') and male characteristics. We measured characteristics of 52 different males of which at least one own brood (i.e. brood produced with the social female) was DNA-sampled. We used multilevel generalized linear mixed models (GLMM) to analyse the effect of male

characteristics on the proportion of EPP in a male's own brood, assuming a binomial error distribution with logit-link function and with the number of offspring in a brood as the denominator. The two-level hierarchical structure of the binomial response data was built up with the male as the highest level and the different broods for each male as the lowest level. This approach allowed us to make full use of all available data, while at the same time accounting for (1) having included individuals more than once, as males often had multiple broods (both within and between seasons), and (2) the number of offspring in a brood, which varied between two and five. The model was implemented by using reweighted iterative generalized least squares (RIGLS) and first-order marginal quasi-likelihood approximation (MQL; [Rasbash et al. 2004](#)). The significance of variables was tested with the Wald statistic, which follows a chi-square distribution.

Second, we tested whether there was a relation between extrapair fertilization success and male characteristics. We defined extrapair fertilization success as the proportion of EPY sired out of the total number of EPY produced in that year ('EPP gained'), using males that were breeding within the study area. As males might have been included more than once if they bred in multiple years, we again used multilevel GLMM, as described above, but now using the total number of EPY as the denominator. The identity of the male was included as the highest level, and the different years of breeding as the lowest level. As males breeding on the edge of the study site (i.e. with at least one adjacent territory that we did not search for nests; 2002: $N = 14$; 2003: $N = 20$) may have sired offspring in nests that we did not sample for DNA, we also repeated this analysis excluding these edge males. Males with EPY mainly sire them in their neighbour's nest ([Bouwman et al. 2006](#)).

Third, we tested whether there was a relation between total fertilization success (i.e. the proportion of offspring sired out of the total number of offspring produced in that year) and male characteristics. The same approach was used as described for extrapair fertilization success (see above). Finally, we directly compared measurements of the cuckolded social male with the measurements of the extrapair male, which both sired offspring in the same brood, in a paired test ('WPmale versus EPmale'; $N = 48$). If more than one extrapair male sired offspring in a brood, we used the average of their measurements in the analysis ($N = 28$). Data collected on male characteristics in 2001–2003 were used in determining age-related characteristics by comparing measurements of males caught in 2 subsequent years. Data from 2002 and 2003 were included in all other analyses. Morphological measurements, plumage characteristics and cloacal protuberance measures were collected in both 2002 and 2003, but in uneven quantities (a maximum of 54 different males were sampled), while song characteristics and age classes were available only in 2003 ($N =$ maximally 30 different males). Several characteristics were intercorrelated ([Table 2](#)).

Within- versus extrapair offspring characteristics

Offspring sex and survival in relation to paternity were analysed with a generalized multilevel linear model,

assuming a binomial error distribution and transformed with the logit-link function. The three-level hierarchical structure of the binomial response data was built up with the male as the highest level, the different broods for each male as the second level and the individual offspring as the lowest level. We analysed morphological differences between within- and extrapair offspring for both maternal and paternal half-siblings by using the last measurements of a brood. These were usually taken at day 7 after hatching (i.e. when the nestlings were ringed; 71%, $N = 80$), but if the last measurements were taken on day 5 or day 6 after hatching (i.e. when a brood was predated before ringing; 13%, $N = 80$) or after day 7 (i.e. when a nest was found with older nestlings; 16%, $N = 80$) these were also included. For maternal half-siblings, comparisons were done within a brood, so correcting for age was not necessary. Paternal half-siblings were compared between broods, so we corrected biometry measurements for age of the nestlings.

For statistical analyses we used SPSS 11.0.1 (SPSS Inc., Chicago, IL, U.S.A.) and MLwiN 2.0 (Centre for Multilevel Modelling, Bristol, U.K.). Nonparametric tests were used in SPSS for data that were not normally distributed. Means are expressed \pm SE, probability values are two tailed and we assumed significance if $P < 0.05$. To control for the chance of making type I errors (i.e. false positives) when performing multiple statistical tests, it has recently been advocated to control the false discovery rate ('FDR'), rather than use (sequential) Bonferroni corrections (Garcia 2004; Verhoeven et al. 2005). When controlling FDR, one controls the proportion of significant results that are in fact type I errors, instead of controlling the chance of making even a single type I error. The latter is done when using a (sequential) Bonferroni correction. The advantage of using FDR over Bonferroni corrections is that it is less restrictive and more powerful. Therefore, after repeated tests in this study, we corrected the significance level α using 'step-up FDR' (Benjamini & Hochberg 1995; Garcia 2004). In short, we ranked the P values in ascending order, j being the resulting rank. We then proceeded from $j = N$ to $j = 1$, until finding a first P value, ranked k , satisfying $P_k \leq k \times \alpha/N$. Then we rejected the null hypothesis H_0 for $j \leq k$ and accepted all the remaining null hypotheses. In the tables we present the original P values that result from the statistical tests, whereas in the text we present the corrected α to control for the proportion of type I errors among significant results.

RESULTS

Male Age and Characteristics

Repeat measurements of several male characteristics (mass (residuals), wing length, tarsus length, badge size, badge colour and cloacal protuberance) between years were available. Within males, there was a significant increase from one year to the next in wing length, badge score and cloacal protuberance (Fig. 1), but not in the other characteristics (all $P > 0.18$). The same result was found for wing length, badge colour and cloacal protuberance when we compared these characteristics in 'young' and 'old'

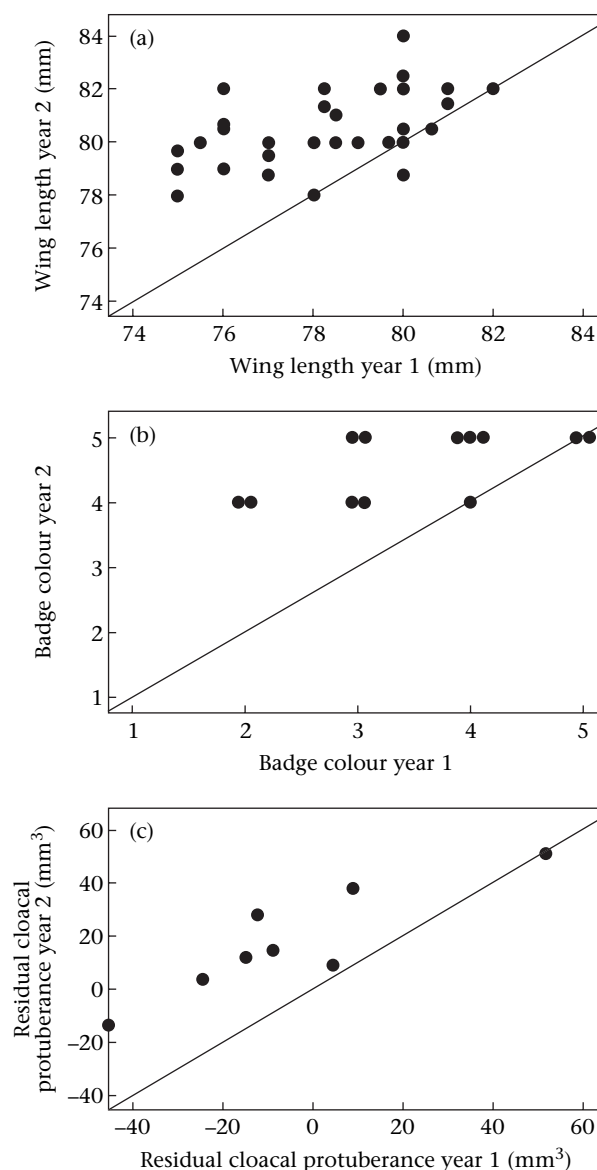


Figure 1. Repeat measurements in successive years for individual males of (a) wing length (paired t test: $t_{35} = -7.71$, $N = 36$, $P < 0.001$), (b) badge colour (1 = brown; 5 = black; $Z = -2.88$, $N = 12$, $P = 0.004$) and (c) cloacal protuberance (residuals over day; $t = -4.63$, $N = 8$, $P < 0.002$). The relation $Y = X$ is indicated by a diagonal line.

males in 2003 (Fig. 2a, b, c). In addition, old males spent more time singing than young males (Fig. 2d). There was a nonsignificant tendency for old males to be heavier than young males (residual mass for young males: -0.18 ± 0.19 , $N = 28$; old males: 0.30 ± 0.21 , $N = 25$; t test: $t_{51} = -1.71$, $P = 0.093$). We found no significant difference between age classes in tarsus length, syllable number, pause length or repertoire size (all $P > 0.31$).

Male Fertilization Success

We found a significant negative relation between a male's wing length and his cuckoldry rate, and

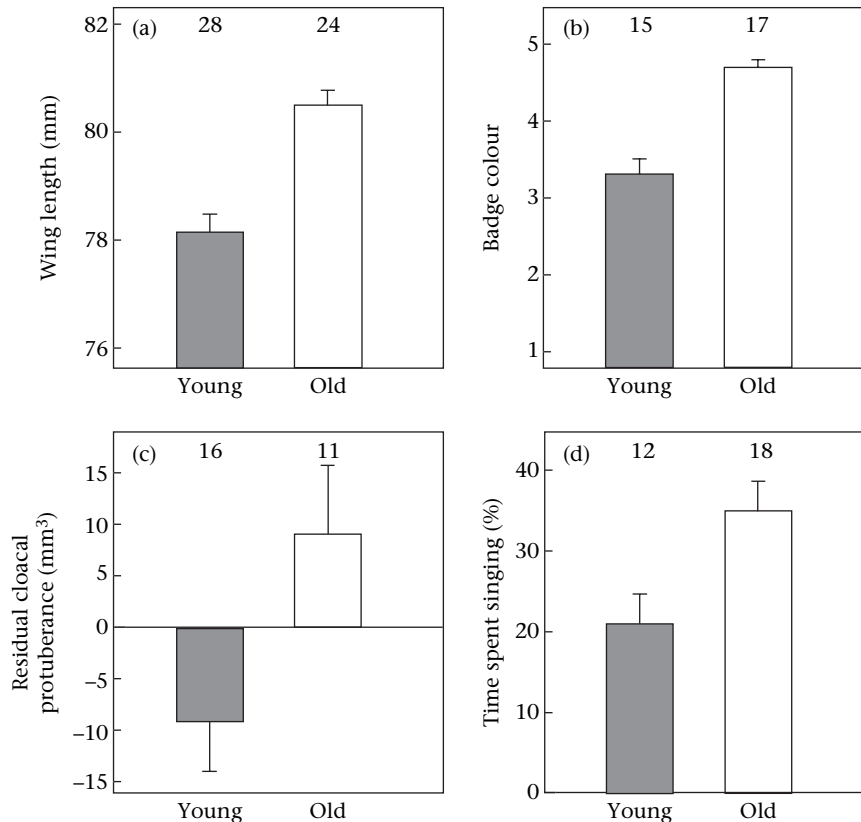


Figure 2. Differences in characteristics ($\bar{X} \pm \text{SE}$) between young and old males for (a) wing length (t test: $t_{50} = -5.22$, $P < 0.001$), (b) badge colour (1 = brown; 5 = black; $t_{30} = -5.92$, $P < 0.001$), (c) cloacal protuberance (residuals over day; $t_{25} = -2.17$, $P = 0.040$) and (d) percentage time spent singing ($t_{28} = -2.61$, $P = 0.014$). 'Old' males were ringed males returning in 2003; 'young' males were unringed. Sample sizes are shown above the bars.

a nonsignificant tendency for a negative effect of both percentage time spent singing and age class (Table 3). However, after correcting the significance level for multiple testing, we found that the relation between a male's wing length and his cuckoldry rate was no longer significant (corrected $\alpha = 0.005$). Cuckoldry rate was not significantly influenced by mass, tarsus length, badge size, badge colour, song quality characteristics or cloacal protuberance size (Table 3). Thus none of the null hypotheses could be rejected.

The characteristics of the extrapair sires were known for 148 extrapair offspring. Males that gained a large proportion of the total number of extrapair fertilizations had longer wings, blacker badges, spent more time singing and were in the 'old' age class (Table 3). All these are characteristics we identified to be strongly related to age (Fig. 2). The proportion of EPP gained was not significantly influenced by mass, tarsus length, badge size, song quality characteristics or cloacal protuberance size (Table 3). As a result of correcting for FDR, the corrected α was set at 0.018 for percentage time spent singing, after which it was no longer significant. Badge colour, wing length and age class remained significant after correction (corrected $\alpha = 0.014$).

Overall, 375 offspring were produced for which we had measured characteristics of the father (2002: $N = 194$;

2003: $N = 181$). Similar to the results found for the proportion of EPP gained, males that gained a large proportion of the total number of fertilizations had longer wings, blacker badges, spent more time singing and were in the 'old' age class (Table 3, Fig. 3). In addition, heavier males tended to sire more offspring, although not significantly so (Table 3). The proportion of total number of fertilizations gained was not significantly influenced by tarsus length, badge size, song quality characteristics or cloacal protuberance (Table 3). However, after correction for FDR, neither percentage time spent singing (corrected $\alpha = 0.018$) nor age class (corrected $\alpha = 0.014$) was still significant. Badge colour and wing length did remain significant after correction (corrected $\alpha = 0.009$). When comparing the total fertilization success of individual males from one year to the next, we found that with an increase in age males produced a larger proportion of the total number of fertilizations in that year (Fig. 4).

Cuckolded and Extrapair Males

Finally, in a direct comparison of characteristics of extrapair males with the characteristics of social males they cuckolded, we found that wing length and age class were significantly different, so that extrapair males had

Table 3. Analysis of the relation between individual male characteristics and fertilization success

Variable	Cuckoldry rate	EPP gained	Total success	WPmale versus EPmale
Mass (residual)	-0.53	+1.45	+3.14	$t=+0.48$
<i>N</i>	52/108	54/73	54/73	48
<i>P</i>	0.47	0.23	0.076	0.64
Wing length	-4.56	+9.02	+18.59	$t=+2.35$
<i>N</i>	52/108	54/73	54/73	48
<i>P</i>	0.033	0.003	<0.001	0.023
Tarsus length	+1.52	+0.97	-0.27	$t=-0.12$
<i>N</i>	52/108	54/73	54/73	48
<i>P</i>	0.22	0.33	0.60	0.91
Badge size	-0.89	+2.33	+1.97	$t=+1.09$
<i>N</i>	52/108	54/73	54/73	48
<i>P</i>	0.35	0.13	0.16	0.28
Badge colour	-0.86	+9.02	+6.79	$Z=+0.97$
<i>N</i>	34/66	36/45	36/45	21
<i>P</i>	0.35	0.003	0.009	0.33
%Time spent singing	-2.86	+4.02	+4.17	$t=+1.52$
<i>N</i>	27/43	30/30	30/30	14
<i>P</i>	0.091	0.045	0.041	0.15
Syllable no.	-1.88	-1.51	-0.95	$t=+0.35$
<i>N</i>	24/39	26/26	26/26	14
<i>P</i>	0.17	0.22	0.33	0.73
Pause length	-0.40	-0.09	-0.22	$t=+0.70$
<i>N</i>	24/39	26/26	26/26	14
<i>P</i>	0.53	0.77	0.64	0.50
Repertoire size	+0.26	-0.57	-0.40	$t=+0.13$
<i>N</i>	24/39	26/26	26/26	14
<i>P</i>	0.61	0.45	0.53	0.90
Age class	-3.00	+11.71	+4.39	$Z=+2.11$
<i>N</i>	24/39	26/26	26/26	17
<i>P</i>	0.083	<0.001	0.036	0.035
Cloacal protuberance	-1.34	+2.34	+2.23	$t=-1.8$
<i>N</i>	39/72	41/49	41/49	25
<i>P</i>	0.25	0.13	0.14	0.084

Fertilization success is divided into (1) cuckoldry rate (i.e. proportion of extrapair paternity (EPP) in male's own brood; sample size (*N*) indicates number of different males/number of broods in years combined), (2) EPP gained (i.e. proportion of EPY gained in other broods; sample size indicates number of different males/number of males in years combined), (3) total success (i.e. proportion of total number of offspring sired; sample size indicates number of different males/number of males in years combined) and (4) direct comparison of cuckolded within-pair male (WPmale) and extrapair male (EPmale; sample size indicates number of broods). Values of statistical tests (i.e. Wald statistics unless stated otherwise) and the directions of the relations are shown. Significant results are highlighted in bold.

longer wings and were older than the cuckolded social males (wing length: social male: 79.2 ± 0.3 mm; extrapair male: 80.0 ± 0.2 mm; age class (1 = 'young'; 2 = 'old'): social male: 1.53 ± 0.12 ; extrapair male: 1.88 ± 0.07 ; Table 3). There was a nonsignificant tendency for extrapair males to have larger cloacal protuberances than the cuckolded social males (residuals over day; 6.0 ± 3.8 versus -4.9 ± 4.1 ; Table 3). There was no significant difference between cuckolded and extrapair males in mass, tarsus length, badge size and colour, song quantity and quality measures (Table 3). However, after correction for FDR, none of the variables above remained significant (corrected $\alpha = 0.009$).

Within- and Extrapair Offspring

There was a significant effect of the interaction between sex and age on mass of the nestlings (Wald $\chi^2_1 = 24.45$, $P < 0.001$) and a near-significant effect of this interaction on tarsus length (Wald $\chi^2_1 = 3.69$, $P = 0.055$) for nestlings between 0 and 10 days after hatching. There was no general bias in sex ratio, as sons and daughters hatched (178 sons, 181 daughters: chi-square test: $\chi^2_1 = 0.03$, $P = 0.87$) and fledged (122 sons, 122 daughters: $\chi^2_1 = 0.0$, $P = 1.0$) in equal numbers. We found no relation between the occurrence of EPP and infertile eggs in a brood (percentage of broods with at least one infertile egg for broods with no EPP: 46%, $N = 26$; broods with EPP: 37%, $N = 70$; $\chi^2_1 = 0.64$, $P = 0.42$). Neither was there a difference in the proportion of infertile eggs in a nest between nests of young (0.18 ± 0.07 , $N = 12$) or old males (0.14 ± 0.04 , $N = 17$; Mann-Whitney U test: $U = 93.5$, $P = 0.68$). However, when including only those nests without EPP, we found that old males had a higher proportion of infertile eggs in their nest (0.26 ± 0.07 , $N = 9$ nests) than young males (0.03 ± 0.03 , $N = 7$ nests; $U = 9.5$, $P = 0.012$).

We found no effect of paternity on sex ratio (including all offspring of known sex and paternity; percentage of males for WPY: 49%, $N = 194$; EPY: 48%, $N = 199$; Wald $\chi^2_1 = 0.04$, $P = 0.83$). There was no difference between within- and extrapair offspring in prehatching mortality (WPY: 4.6%, $N = 194$; EPY: 5.0%, $N = 199$; Wald $\chi^2_1 = 0.09$, $P = 0.77$) or in prefledging mortality (WPY: 5%, $N = 132$; EPY: 10%, $N = 137$; Wald $\chi^2_1 = 0.47$, $P = 0.49$). Recruitment rates were low: on average 5% ($N = 173$) of offspring that fledged in 2001 and 2002 bred in our study site the subsequent year. Daughters recruited at a significantly lower rate (1%, $N = 90$) than sons (8%, $N = 91$; chi-square test: $\chi^2_1 = 4.64$, $P = 0.031$). Only one (extrapair) daughter and seven sons recruited in our population; of the latter, four were extrapair.

We compared mass, tarsus, internal relatedness and mean d^2 between maternal half-siblings in 34 broods of mixed paternity. As WPY and EPY did not differ in sex ratio, we included both sexes together in the analysis. EPY had longer tarsi than their within-pair half-siblings (Table 4). There was no difference in mass, internal relatedness or mean d^2 between maternal half-siblings (Table 4). The difference in tarsus length remained significant after we controlled for FDR (corrected $\alpha = 0.013$). There were 28 males for which measurements were available of both WPY in their own nests and EPY in other nests. We found no difference in mass, tarsus, internal relatedness or mean d^2 when comparing these paternal half-siblings (mass and tarsus corrected for sex and age; Table 4).

DISCUSSION

Male Characteristics

A male's fertilization success can be determined at three levels: (1) the proportion of paternity lost to other males in his own brood; (2) the proportion of EPFs gained in other broods; and (3) the proportion of total fertilizations

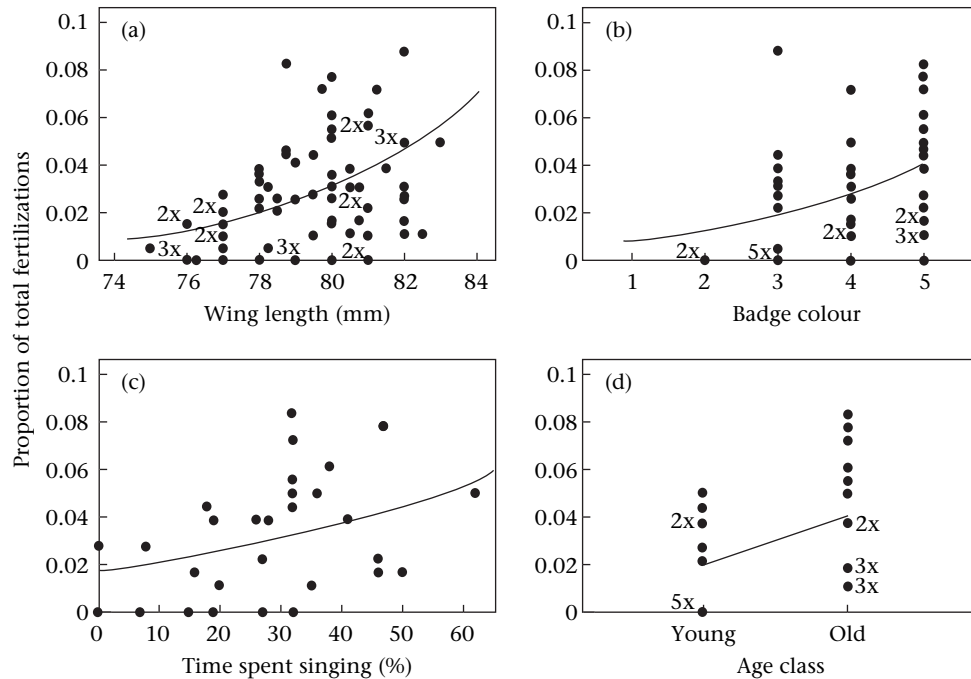


Figure 3. Relation between total fertilization success (i.e. the proportion of total number of offspring sired) and male characteristics: (a) wing length, (b) badge colour (1 = brown; 5 = black), (c) percentage time spent singing and (d) age class. 'Old' males were ringed males returning in 2003; 'young' males were unringed. For other details see Table 3.

gained, both in his own and in other broods. In this study we have shown that male extrapair and total fertilization success in reed buntings is predicted by characteristics related to male age. In addition, male within-pair fertilization success showed a similar pattern, although this was not significant after we corrected for multiple testing. Using a within-male comparison, we previously found that with an increase in age, individual males significantly increased their fertilization success in their own brood (Bouwman & Komdeur 2005). The strongest test of male success is a direct comparison of the social male and the extrapair male by which he is cuckolded. We found indications, although again nonsignificant after we corrected for multiple testing, that extrapair males had longer wings and were older, suggesting that age is a predictor of male success.

This finding is in agreement with the predictions of the good genes (Kempnaers & Dhondt 1993) and fertility insurance hypotheses (Sheldon 1994), but not necessarily with the genetic compatibility hypothesis (Griffith et al. 2002). Older males will on average be of higher quality than younger males, as they have shown to be able to survive longer (Trivers 1972; Manning 1985; Kokko & Lindstrom 1996; but see Hansen & Price 1995). If females seek viability genes, they may benefit from selecting older males. However, if male functional fertility improves with male age, then females seeking EPFs to insure against infertility would generate a similar association between male age and fertilization success as females seeking viability genes for their offspring (Sheldon 1994). The age-related increase in size of the cloacal protuberance may seem consistent with Sheldon's (1994) suggestion of a higher functional fertility of older males as a result of

higher sperm storage capacities (Nakamura 1990; Birkhead et al. 1993), although high sperm storage capacity does not say anything about sperm quality. Since the size of the cloacal protuberance had no significant effect on male fertilization success, in our study, it is unlikely that older males do indeed have a higher functional fertility. Females seeking compatible genes (Tregenza & Wedell 2000) are not expected to benefit from selecting older males, as the benefits of the combination of paternal and maternal genes do not change with age.

In many species, males show age-related differences in plumage (e.g. Sundberg & Dixon 1996; Richardson & Burke 1999), providing females with a cue to judge male age. In reed buntings, females may use badge colour and the percentage of time spent singing as an indication of male age, at least as being either young (1 year old) or old (>1 year old), as these variables increased with male age. In agreement with our finding that the percentage of time spent singing is related to male age, males with longer wings also spent a larger percentage of time singing in an English population of reed buntings (O'Malley 1993). Although wing length and cloacal protuberance size also increased with age, it is unlikely that they are used as cues to judge age. The directions of the relations between age-related characteristics and fertilization success were identical, but not all characteristics related to age predicted fertilization success on each level of analysis with similar strength. Apparently these characteristics differ in importance when determining within- or extrapair fertilization success. In particular, the weak relation between cloacal protuberance size and fertilization success compared to the other age-related factors was surprising. This discrepancy may be caused by the lower

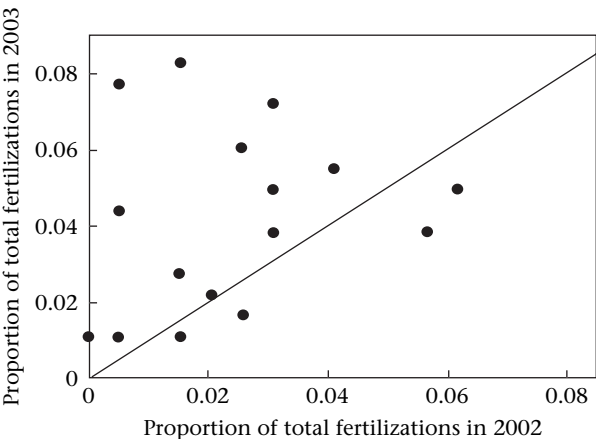


Figure 4. Repeat measurements in successive years (i.e. 2002 and 2003) of proportion of total fertilizations for individual males (paired t test: $t_{15} = -2.64$, $P = 0.019$). The relation $Y = X$ is indicated by a diagonal line.

strength of the relation between age and cloacal protuberance size.

Males that sired both more extrapair offspring in other broods and more offspring in total were older, and had longer wings and blacker badges. The effect of age-related characteristics on within-pair fertilization success was less strong than on extrapair fertilization success. In other species, older males that were found to be more successful in siring offspring in their own broods were often also more successful in siring extrapair offspring (reviewed in Griffith et al. 2002). However, being successful in siring within-pair offspring is not a prerequisite for being successful in siring extrapair offspring or vice versa (reviewed in Griffith et al. 2002). Owing to intercorrelations between the age-related characteristics, we are unable to evaluate the relative importance of male traits in our study and are only confident to state an overall importance of age. To disentangle the relative importance of each of these different factors related to age, an experimental set-up is required. Whether males are more successful because they are older, or for instance because they have blacker badges, remains to be determined.

As reed buntings are highly sexually dimorphic, we were surprised that neither badge size nor our measures of song quality significantly affected male fertilization success. Plumage ornaments and song quality significantly predict fertilization success in many species (reviewed in Griffith et al. 2002). Possibly these characteristics are more important for intrasexual than for intersexual signalling in reed buntings (e.g. Møller 1987).

An interesting question that remains is which sex initiates extrapair mating behaviour in the reed bunting. Older males may be more successful through different, not mutually exclusive, mechanisms: (1) females may actively select older males (e.g. in Bullock's orioles, *Icterus bullockii*: Richardson & Burke 1999), or (2) older males may be more persistent or better able to exploit EPC opportunities (e.g. in red-winged blackbirds, *Agelaius phoeniceus*: Weatherhead & Boag 1995). Male reed buntings were often seen intruding in neighbouring territories, especially during the fertile period of the female, whereas females were not seen making extraterritorial forays or actively soliciting EPCs (Marthinsen et al. 2005; personal observation). Buchanan (2001), however, described seeing two female reed buntings actively solicit EPCs on neighbouring territories. Owing to the secretive nature of females, we cannot exclude the possibility that this occurs in our population, or that females solicit copulations from intruding males. The occurrence of EPP is the result of an interaction between social male, social female and extrapair male, and the fitness of all three depends on the behaviour of the others (reviewed in Westneat & Stewart 2003). More behavioural observations are needed on interactions between the participants in extrapair mating behaviour in the reed bunting.

Within- and Extrapair Young

One way to distinguish between the three female benefit hypotheses is to determine differences between within- and extrapair young (Sheldon 1994; Griffith et al. 2002). If females seek insurance against infertility through EPCs, WPY and EPY are not expected to differ in fitness, whereas if genetic benefits are driving extrapair mating behaviour, then EPY are expected to be of higher fitness than

Table 4. Paired comparison of characteristics between maternal half-siblings in broods of mixed paternity ($N = 34$), and between paternal half-siblings in different broods ($N = 28$)

Variables	WPY	EPY	t	P
Maternal half-siblings				
Tarsus length (mm)	17.6±0.3	18.0±0.3	-2.62	0.013
Mass (g)	13.6±0.5	13.8±0.4	-0.89	0.38
Internal relatedness	-0.06±0.02	-0.03±0.02	-1.01	0.32
Mean d^2	861±72	788±75	0.97	0.34
Paternal half-siblings				
Residual tarsus length	0.12±0.08	0.09±0.09	0.23	0.82
Residual mass	0.09±0.15	0.06±0.14	0.14	0.89
Internal relatedness	-0.04±0.02	-0.02±0.03	-0.72	0.48
Mean d^2	877±86	885±81	-0.08	0.94

Mean values ± SE for within-pair young (WPY) and extrapair young (EPY) are presented. Significant variables are highlighted in bold.

WPY. To support both the good genes and the genetic compatibility hypotheses, EPY are expected to be fitter than their maternal half-siblings. Depending on the degree of compatibility between males siring EPY and their own social females, the genetic compatibility hypothesis may also predict that EPY are fitter than their paternal half-siblings.

In a Norwegian population of reed buntings, no differences were found between within- and extrapair nestlings in immunocompetence, growth rate, condition (Kleven & Lifjeld 2004) or heterozygosity (Kleven & Lifjeld 2005). In our study, we found no differences in survival, internal relatedness, mean d^2 and fledgling mass. However, we did find that EPY had significantly longer tarsi than their maternal half-siblings, a trend also found in a reed bunting population in Switzerland (M. Kaiser & S. Suter, unpublished data). This difference in tarsus length may be a result of several mechanisms, such as good or compatible paternal genes, differential provisioning behaviour to WPY or EPY, or EPY hatching from larger eggs or earlier than WPY.

In blue tits and red bishops, *Euplectes orix*, successful males have longer tarsi than unsuccessful males (Kempenaers et al. 1997; Friedl & Klump 2002). In several species, including blue tits, tarsus length has been found to be heritable (e.g. Dhondt 1982; Smith 1993). However, although extrapair blue tit nestlings had on average longer tarsi than their within-pair half-siblings, this difference was not significant (Kempenaers et al. 1997). We have shown that male reed buntings with longer tarsi were not more successful in siring offspring, and thus it is not likely that extrapair offspring have long tarsi just because their fathers do as well. However, it may be possible that because of good or compatible paternal genes, extrapair young develop faster than their maternal half-siblings (Neff 2004).

Tarsus growth is influenced by the provisioning behaviour of the parents (e.g. Alatalo & Lundberg 1986; Moreno et al. 1997); possibly, females provide more food to extrapair nestlings, thereby enhancing the growth of tarsi. This is, however, unlikely in reed buntings because males and females provision at similar rates to individual within- and extrapair offspring in broods of mixed paternity (Bouwman et al. 2005).

Alternatively, EPY may hatch from larger eggs (Cunningham & Russell 2000), or hatch earlier. In the latter case EPY would have longer tarsi around day 7, just because they are slightly older. Early hatching chicks may have developed faster in the egg, because of superior genes or maternal investment (Sheldon 2000), or come from eggs produced early in the laying sequence. Unfortunately, we do not have any data on the exact laying or hatching order of WPY and EPY in reed buntings. Previous studies on the relation between EPP and laying or hatching order have generated mixed results, since more EPY were found early in the laying sequence by Cordero et al. (1999), late in the sequence by Riley et al. (1995) and Dunn et al. (1999) and randomly distributed by Westneat et al. (1995) and Whittingham et al. (2003). If early hatching extrapair offspring have a fitness benefit, females would also gain genetic benefits for their offspring

through extrapair matings, although these might not have been apparent in our short-term measures of fitness.

Apart from the difference in tarsus length, we found no other support for the genetic compatibility hypothesis. There was no significant difference in internal relatedness or mean d^2 between maternal half-siblings from the same brood. Furthermore, we found no indication that there was a difference in measurements between paternal half-siblings, although in this case we could not account for any maternal or environmental differences, besides provisioning rates, between within- and extrapair broods of the same male. In addition, in a Norwegian population of reed buntings no difference was found in immunocompetence between paternal half-siblings (Kleven & Lifjeld 2004). However, as data on long-term survival and reproductive success are still lacking in reed buntings and our sample size was relatively small compared to the study by Foerster et al. (2003) ($N = 101$ broods of mixed paternity), we cannot exclude the possibility that females do gain genetic benefits through extrapair matings.

It appears unlikely that females seek insurance against infertility of their eggs by gaining EPCs with older males, for the following three reasons. First, there was no significant effect of cloacal protuberance size on fertilization success. Second, unlike Wetton & Parkin (1991) and Gray (1997), we did not find a relation between the hatching success of a brood and the occurrence of EPP in that brood. Third, there was no difference in the proportion of infertile eggs between nests of young and old males when including all nests. When including only nests containing no EPP, nests of old males showed a higher proportion of infertile eggs than nests of young males. Possibly as a consequence of higher extrapair mating activity, old males may be more prone to sperm depletion, and thus to having infertile eggs in their nests, than young males. Alternatively, sperm quality may decrease with male age because of a senescence effect. However, this does not appear likely since older males are successful at gaining EPFs.

Conclusion

Older males were more successful than younger males in gaining fertilizations in other broods, but were not significantly more successful in gaining fertilizations in their own brood. When investigating differences between WPY and EPY, we found that EPY had longer tarsi at day 7 than WPY, but did not differ in any of the other measured characteristics. So far, it is unclear whether this difference in tarsus length is related to a difference in fitness benefits between maternal half-siblings. Without long-term data on fitness benefits, we cannot yet exclude the possibility that female reed buntings gain genetic benefits for their offspring through extrapair matings. However, it is unlikely that extrapair matings with older males provide females with insurance against infertility of their eggs.

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